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Progress has been made on identifying biosensors that will be used to report on the fermentation yields of industrially relevant biological compounds. About half of the desired chemicals have already been screened. Enrichment cultures were used to isolate microbes that utilize these compounds as their sole carbon and energy source. The results are better than predicted and easily surpass the first milestone of approximately 50 microbes growing on at least 10 different biochemicals. The current results substantiate a minor conjecture of the proposal and indicate that any chemical made by one organism is likely to be used as food by another. In preparation for sequencing and biosensor elucidation, we recommend that growth screens continue as forecasted in the proposal.

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J. Craig Venter INSTITUTE

Rapid parallel screening for strain optimization

Report Title:	Quarterly R&D Status Report
Report Number:	HR0011-12-C-0062.1
Reporting Period:	May 17, 2012 to August 16, 2012
Contract No.:	HR0011-12-C-0062
Performing Organization:	J. Craig Venter Institute 9704 medical Center Drive Rockville, MD 20850 USA
Principal Investigator:	Chuck Merryman

Abstract

Progress has been made on identifying biosensors that will be used to report on the fermentation yields of industrially relevant biological compounds. About half of the desired chemicals have already been screened. Enrichment cultures were used to isolate microbes that utilize these compounds as their sole carbon and energy source. The results are better than predicted and easily surpass the first milestone of approximately 50 microbes growing on at least 10 different biochemicals. The current results substantiate a minor conjecture of the proposal and indicate that any chemical made by one organism is likely to be used as food by another. In preparation for sequencing and biosensor elucidation, we recommend that growth screens continue as forecasted in the proposal.

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Summary

Work was initiated on the project on May 17, 2012. In this quarter, progress has been made on identifying biosensors that will be used to report on the fermentation yields of industrially relevant biological compounds. Numerous microbes that utilize such compounds as their sole carbon source have been isolated. The results are better than predicted and easily surpass the first milestone as well as indicating that any chemical made by one organism is likely to be used as food by another. Screening of additional biochemicals should continue as planned so that it is possible to accurately estimate how frequently growth is dependent on a microbes ability to sense a particular compound.

Fifty-three compounds have been paired with more than 90 microbes as suggested by colony morphology (see below). We have completed clonal purification and generated freezer stocks for 49 colonies that utilize 31 biochemicals. Archival stocks are being made for additional colonies and the preparation of genomic DNA will begin soon.

Twenty chemical compounds from the initial set remain to be examined. Currently, enrichment cultures for these chemicals are being processed using microbes from a manure pile and a pond. This set of 20 chemicals is more cumbersome than many of those already processed due to poor water solubility, etc. Nevertheless, about half have already produced substantial growth. Moreover, limonene, tocopherol, farnesene, etc are compounds of a similar nature and exploratory culturing schemes designed to address cumbersome physicochemical properties yielded archived clones. Thus, we are confident that a handful of straightforward culturing techniques will be sufficient to search for sensors that respond to most biological chemicals. Ultimately, a repechage scheme focusing on microbes from novel environmental sites will be used to re-examine compounds where no archival clones are available upon completion of the first round of screening.

Seventy-eight of the 100 or so desired biochemicals have been identified and purchased. An additional 140 candidates have been gleaned from the literature. We anticipate that a relatively small number of these molecules will survive down-selection criteria such as industrial potential, verified biosynthetic pathway, etc. In the next few weeks, we aim to select the remaining 20 or so biochemicals that are desired.

Introduction

The overall goal in this contract is to link cell-based production to cell survival and thereby make the engineering of new microbial strains that produce industrially relevant biochemicals routine. Recent synthetic biology techniques can make billions of variant cells. Although, many potentially

informative mutants are easily made, product yield can only be determined in a few of these. The majority of industrially relevant biomolecules are not chromophores, naturally discernible, or otherwise easily detected. Nevertheless, genetic circuits are capable of linking chemical production to discernible signals such as growth or color intensity. Such a system would allow numerous mutants and mutant combinations to be examined quickly. Genetic circuits needed to screen mutant populations in parallel rely upon the the availability of an appropriate biosensor that activates a reporter gene in a product dependent fashion. In this project, genes for two-component and one-component signaling systems (that respond to industrially relevant biomolecules) are identified using microbial growth assays, sequencing, and quantitative PCR (qPCR). To demonstrate that such sensors can be used to maximize product yield, one sensing system will be further engineered. We will reformat this sensor so that it drives expression of a reporter such as an antibiotic resistance marker. This sensor/resistance cassette, and a biosynthetic pathway capable of producing the molecule to which the sensor responds, will be placed within a heterologous host that does not have an overlapping pathway. Basal synthesis of the targeted chemical (by the orthogonal biosynthetic pathway) activates the sensor and increases transcription of the resistance marker (i.e. reporter). In other words, the fermentation product is also the sensor ligand and thus, biosynthesis drives production of the reporter and cell survival. Antibiotic levels in the media will be adjusted so that basal product yield, and hence basal marker activity, is insufficient for survival. Targeted, genome-wide and barcoded alterations to the host genome will then be installed. Variants with better and better chemical production survive by virtue of their ability to withstand increasing antibiotic challenge.

Methods, Assumptions and Procedures

Although several alternative approaches were initially examined for isolating microbes, iterative enrichment cultures provided the fastest and most general approach with the least background. Well composted manure samples and pond sediment were diluted 1:5 (w/w) with water and extensively macerated with a spatula. Heavy material was allowed to settle to the bottom of the tube. The aqueous layer was removed and diluted 100-fold with water. Ten microliter aliquots were then distributed in 15 ml culture tubes. Enrichment broths were made using 0.1 percent carbon source in M9 mineral salts media. One-hundred microliters of a 1% solution of the target biochemical (i.e. carbon source) was added to 0.9 ml of M9 salts. A one-percent slurry was used for insoluble solids. Insoluble liquids were used neat and 100 microliters was applied to the mineral salts solution. Tubes containing volatile compounds were sealed with screw-capped lids and opened occasionally. Enrichment cultures were incubated at room temperature and shaken at 200 rpm for 60 hours. Two-microliters of each culture were withdrawn and directly pipetted on the top of a glass bead in each sector of a square culture plate comprised of 48 individual wells. These trays were shaken vigorously to distribute microbes over the media surface. Solid media contained Luria broth, glucose, glycerol, and succinate. As colonies developed they were picked and transferred to a second and third enrichment culture containing the appropriate carbon source and M9 mineral salts. Archived versions were stored at -80C in broth or broth with 15% glycerol. Finally, agar plates using the carbon source and mineral media were also generated, sealed and stored at 4C. Slurry's were formed within the media and insoluble liquids were spread on the surface of plates used for storage.

Results and Discussion

More than 90 microbes using 53 biochemicals as their sole carbon and energy source have been isolated and are being archived (a large file with pictures of the colonies is available upon request). Enrichment cultures are easily processed and after 3 rounds of ~1000-fold dilution and growth, this technique ensures that isolated colonies utilize the desired carbon source. In essence, the initial material is diluted more than a billion-fold. Intervening colony growth on solid media supplemented with glucose, glycerol and succinate ensures that the selected colonies also replicate on standard carbon sources. Thus, differential expression of transcription factors and similar genetically encoded sensors can be evaluated to determine which ones within a genome are up-regulated in response to the target biochemicals. Thus, the results so far suggest that we

will likely be able to identify a selection of sensors to include in genetic circuitry that reports on product yields.

Conclusions

The results indicate that any chemical made by one organism is likely to be used as food by another. Microbes typically utilize the most efficient carbon source available (glucose often being the preferred substrate). More exotic carbon sources are generally subject to catabolite repression and systems for their utilization are activated after preferred carbon sources are exhausted. Besides catabolite repression, sensors are often employed so that a microbe can activate the appropriate degradation pathway for a non-preferred carbon source. Nevertheless, how often sensor systems are used cannot currently be estimated because negative results rarely appear in the literature. The variety of carbon sources (i.e. biochemicals) that support robust growth in our initial assays suggests that sensors which respond to a wide range of biochemicals may be readily available in the environment. Now that this point has been established it is reasonable to use low cost next-generation sequencing approaches and functional genomics to unequivocally identify sensors and how often they are employed for growth on non-standard carbon sources. This will set the stage for downstream work on the overproduction of such molecules.

Planned Activities for the Next Reporting Period

During the next reporting period we will continue to isolate microbes that grow on our selection of biochemicals. We expect that a sufficient set will be obtained before the quarter is complete. In such a case, we will begin preparing chromosomal DNA from isolated colonies in preparation for phylotyping, sequencing and similar objectives slated for the third quarter.

Program Financial Status

	Planned Expend	Actual Expend (Cumulative to Date)	% Budget Completion	At Completion	Latest Revised Estimate	Remarks
Task 1	$91,978.50	$28,060.98	N/A*	N/A*	$50,000	N/A
Cumulative	$367,914.00	$28,060.98	7.6%	N/A	$367,914.00	N/A

*Expenses for the month of July are not yet available due to the timing of this report.

There is no management reserve or unallocated resources.

Based on the currently authorized work:

- Is current funding sufficient for the current fiscal year? Yes
- What is the next fiscal year funding requirement at current anticipated levels? The budgeted amount for Year 2 of the project is $396,905.25.
- Have you included in the report narrative any explanation of the above data and are they cross-referenced? Not applicable; current funding is sufficient for the current fiscal year.